CNRE BINDING FACTORS AND USES THEREOF

Government Support

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Related Applications

This application claims priority under 35 USC § 119(e) from U.S. Provisional Patent Application Serial No. 60/082,997 filed on April 24, 1998, entitled NRE BINDING FACTORS AND USES THEREOF. The contents of the above-identified application is hereby expressly incorporated by reference.

Field of the Invention

This invention relates to nucleic acids and encoded polypeptides of Regulatory Element Binding factors (REBs), and diagnostics and therapeutics related to medical conditions associated with such genes and polypeptides, including hypertension and cancer.

Background of the Invention

Hypertension is probably the most important public health problem in developed countries. It is common, asymptomatic, readily detectable, and often leads to lethal complications if left untreated. Although our understanding of the pathophysiology of an elevated arterial pressure has increased, in 90-95% of cases the etiology (and thus potentially the prevention or cure) is still largely unknown. As a consequence, in most cases, the hypertension is treated nonspecifically, resulting in a large number of minor side effects and a relatively high (approximately 50%) noncompliance rate.

Patients with arterial hypertension and no definable cause are said to have primary, essential, or idiopathic hypertension. Approximately 15% of patients with essential hypertension have plasma renin activity levels elevated above the normal range. It has therefore been suggested that plasma renin plays an important role in the pathogenesis of the elevated arterial pressure in these patients.

Renin is a proteolytic enzyme (aspartyl proteinase) that is produced and stored in the granules of the juxtaglomerular cells surrounding the afferent arterioles of glomeruli in the

kidney. Renin acts on the basic substrate angiotensinogen (a circulating α_2 globulin made in the liver) to form the decapeptide angiotensin I. Angiotensin I is then enzymatically transformed by a converting enzyme present in many tissues, particularly in the pulmonary vascular endothelium, to the octapeptide angiotensin II. Angiotensin II is a potent pressor agent and exerts its action by a direct effect on arteriolar smooth muscle. In addition, angiotensin II stimulates production of aldosterone by the zona glomerulosa of the adrenal cortex. Currently, some of the most popular drug therapies for hypertension target this renin-angiotensin pathway. An example of such group of drugs includes the angiotensin converting enzyme (ACE) inhibitors. These drugs inhibit the enzyme converting angiotensin I into angiotensin II. Although effective in treating hypertension, this and other categories of antihypertensive drugs are often associated with a number of side effects.

Studies in the mouse have shown that renin exhibits tissue specific expression. In the mouse renin is encoded by two loci (*Ren1*^{d} and *Ren2*^{d}) is expressed in a variety of extrarenal tissues such as the submandibular gland (SMG), adrenal, heart, testis and ovary.

Tissue-specific gene expression is dependent on the specific interaction of *cis*-DNA sequences with nuclear *trans*-activating proteins. Certain *trans*-acting factors are ubiquitous, whereas others exhibit temporal, tissue, and/or cell specific expression. Others have shown that the interaction of a negative regulatory element (NRE or silencer) with specific nuclear *trans*-acting proteins plays an important role on tissue-specific regulation of gene expression. (Williams, TM, et al., *Science*, 1991, 254:1791-1794; Laden, JM, *Annu Rev Immun*, 1993, 11:539-570). Further complexity is obtained when multiple *trans*-acting factors, especially those exerting opposing effects, are able to bind to the same *cis*-element. In this case, competition from the same element can play an important role in gene regulation.

Since renin is the rate-limiting enzyme regulating the formation of angiotensin II and the initiation of the renin-angiotensin system cascade, factors that regulate renin expression would prove very useful in alleviating hypertension.

We have previously demonstrated the existence of a CNRE element in the *Ren1*^{d} gene. (Nakamura, N., et al., *Proc Natl Acad Sci USA*, 1989, 86:56-59). This CNRE element was later characterized to be a ~30 base pair DNA fragment. (Barrett, G., et al., *Proc Natl Acad Sci USA*, 1992, 89:885-889). Such a CNRE motif also occurs in the promoter regions found in various genes including human renin, collagen II, the T-cell receptor and c-myc.

There is a need to ameliorate the course of hypertension and other cardiovascular diseases.

There also exists a need to identify the genes responsible for hypertension, cardiovascular diseases, cancer and other disorders.

An object of the invention is to provide compounds that desirably influence the course of hypertension and cancer.

Another object of the invention is to provide therapeutics for treating diseases or conditions involving the CNRE and CNRE binding factors.

Still another object of the invention is to provide diagnostics and research tools relating to hypertension, cardiovascular diseases, and other disorders. These and other objects will be described in greater detail below.

Summary of the Invention

The invention relates to CNRE-Binding factor polypeptides (CNREBs), nucleic acids encoding such polypeptides, and fragments and biologically functional variants thereof. The invention also provides expression vectors containing those molecules and host cells transfected with those molecules. The invention also pertains to therapeutic and diagnostic methods related to the foregoing polypeptides and nucleic acids, as well as compositions for therapeutic and diagnostic uses. Also included in the invention are agents which bind the foregoing CNREB polypeptides and nucleic acids.

The invention in one aspect involves the cloning of cDNAs encoding CNRE-binding transcriptional factors. CNRE-binding transcriptional factors bind to any of the sequences set forth in SEQ ID NO:21, SEQ ID NO:22, or SEQ ID NO:23, commonly present in the 5'-flanking region (otherwise known as the promoter) of a number of genes including the human, mouse and rat renin genes, the human c-myc gene, collagen Type II , and the T cell Receptor, and modulate the expression of these genes.

According to one aspect of the invention, isolated nucleic acid molecules that code for a CNREB-2 polypeptide are provided and include: (a) nucleic acid molecules which hybridize under stringent conditions to a molecule selected from the group consisting of the nucleic acid of SEQ ID NO:7, the nucleic acid of SEQ ID NO:17, and the nucleic acid of SEQ ID NO:19, and which code for a CNREB-2 polypeptide; (b) deletions, additions and substitutions of (a) which code for a respective CNREB-2 polypeptide; (c) nucleic acid molecules that differ from the nucleic acid molecules of (a) or (b) in codon sequence due to the degeneracy of the genetic code; and (d) complements of (a), (b) or (c). In certain embodiments, the isolated nucleic acid

molecule comprises SEQ ID NO:17. In some embodiments the isolated nucleic acid molecules are those comprising the human cDNA or gene corresponding to SEQ ID NO:17. The isolated nucleic acid molecule also can comprise a molecule which encodes the polypeptide of SEQ ID NO:18 and has CNREB-2 activity. Preferably the isolated nucleic acid molecule consists of the nucleotide sequence of SEQ ID NO:17.

The invention in another aspect provides an isolated nucleic acid molecule selected from the group consisting of: (a) a unique fragment of nucleic acid molecule selected from the group consisting of the nucleic acid of SEQ ID NO:7, the nucleic acid of SEQ ID NO:17, and the nucleic acid of SEQ ID NO:19, (b) complements of (a), provided that the unique fragment includes a sequence of contiguous nucleotides which is not identical to any sequence selected from the sequence group consisting of: (1) sequences described in SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16, (2) complements of (1), and (3) fragments of (1) and (2).

In one embodiment, the sequence of contiguous nucleotides is selected from the group consisting of (1) at least two contiguous nucleotides nonidentical to the sequence group, (2) at least three contiguous nucleotides nonidentical to the sequence group, (3) at least four contiguous nucleotides nonidentical to the sequence group, (4) at least five contiguous nucleotides nonidentical to the sequence group, (5) at least six contiguous nucleotides nonidentical to the sequence group, (6) at least seven contiguous nucleotides nonidentical to the sequence group.

In another embodiment, the fragment has a size selected from the group consisting of at least: 8 nucleotides, 10 nucleotides, 12 nucleotides, 14 nucleotides, 16 nucleotides, 18 nucleotides, 20, nucleotides, 22 nucleotides, 24 nucleotides, 26 nucleotides, 28 nucleotides, 30 nucleotides, 40 nucleotides, 50 nucleotides, 75 nucleotides, 100 nucleotides, 200 nucleotides, 1000 nucleotides and every integer length therebetween.

According to another aspect, the invention provides expression vectors, and host cells transformed or transfected with such expression vectors, comprising the nucleic acid molecules described above.

According to another aspect of the invention, an isolated polypeptide is provided. The isolated polypeptide is encoded by the foregoing isolated nucleic acid molecules of the invention. In some embodiments, the isolated polypeptide is encoded by the nucleic of SEQ ID NO:7, giving rise to a polypeptide having the sequence of SEQ ID NO:8 that has CNREB-2 activity. In other embodiments, the isolated polypeptide is encoded by the nucleic of SEQ ID NO:19, giving

rise to a polypeptide having the sequence of SEQ ID NO:20 that has CNREB-2 activity. In preferred embodiments, the isolated polypeptide is encoded by the nucleic of SEQ ID NO:17, giving rise to a polypeptide having the sequence of SEQ ID NO:18 that has CNREB-2 activity. In further embodiments, the isolated polypeptide may be a fragment or variant of the foregoing of sufficient length to represent a sequence unique within the human genome, and identifying with a polypeptide that has CNREB-2 activity, provided that the fragment includes a sequence of contiguous amino acids which is not identical to any sequence encoded for by the nucleic acid sequence as described in SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16. In another embodiment, immunogenic fragments of the polypeptide molecules described above are provided.

According to another aspect of the invention, isolated binding polypeptides are provided which selectively bind a polypeptide encoded by the foregoing isolated nucleic acid molecules of the invention. Preferably the isolated binding polypeptides selectively bind a polypeptide which comprises the sequence of SEQ ID NO:18, or fragments thereof. In preferred embodiments, the isolated binding polypeptides include antibodies and fragments of antibodies (e.g., Fab, $F(ab)_2$, Fd and antibody fragments which include a CDR3 region which binds selectively to the CNREB-2 polypeptide). In certain embodiments, the antibodies are human.

According to another aspect of the invention, a method for isolating nucleic acid molecules encoding polypeptides having CNREB-2 activity, are provided. The method involves: (a) providing a nucleic acid molecule comprising SEQ ID NO:17, or a fragment of at least 8 contiguous nucleotides thereof; (b) using the nucleic acid molecule of (a) as a probe to obtain under stringent screening conditions a nucleic acid molecule encoding a polypeptide which is a candidate having CNREB-2 activity; (c) expressing in a host cell the isolated nucleic acid molecule obtained in (b) to generate the polypeptide; and (d) measuring CNREB-2 activity of the expressed polypeptide, the presence of such activity being indicative of an isolated nucleic acid encoding a polypeptide with CNREB-2 activity.

According to another aspect of the invention, methods for decreasing renin expression in a mammalian cell which expresses renin are provided. The methods include contacting the mammalian cell with a CNREB-1 inhibitor in the cell in an amount effective to decrease renin expression in the mammalian cell. In some embodiments, the step of contacting the mammalian cell with a CNREB-1 inhibitor occurs *in vitro* or *in vivo*. CNREB-1 inhibitors include antisense CNREB-1 nucleic acids, dominant negative CNREB-1 nucleic acids, dominant negative

CNREB-1 polypeptides, anti-CNREB-1 antibodies, anti-CNREB-1 antibody fragments, and polypeptides and functional fragments or variants thereof which inhibit CNREB-1 activity. In certain embodiments, the CNREB-1 inhibitor is a nucleic acid. In preferred embodiments, the nucleic acid is an antisense nucleic acid or a dominant negative nucleic acid of a CNREB-1 nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In some embodiments the CNREB-1 inhibitor is not an adenylate cyclase inhibitor. Any of the foregoing embodiments, however, may further comprise co-administration of an adenylate cyclase inhibitor in an amount effective to decrease renin expression in the mammalian cell.

According to yet another aspect of the invention, a method for decreasing CNREB-1 activity in a subject, is provided. The method involves administering to a subject in need of such treatment a CNREB-1 inhibitor in an amount effective to decrease CNREB-1 activity in the subject. In some embodiments, the amount is sufficient to decrease CNREB-1 activity below normal baseline levels. In certain embodiments of the foregoing methods, the subject has an elevated risk of hypertension, has hypertension, has an elevated risk of myocardial infarction, has an elevated risk of an ischemic stroke, has experienced an ischemic stroke, has an elevated risk of a condition selected from the group consisting of progressive renal disease, nephropathy, congestive heart failure, coronary artery disease, optic disc swelling, and coarctation of the aorta, or has a condition selected from the group consisting of progressive renal disease, nephropathy, congestive heart failure, coronary artery disease, optic disc swelling, and coarctation of the aorta. In certain embodiments, the CNREB-1 inhibitor is administered acutely. In other embodiments the agent is administered CNREB-1 inhibitor. In still further embodiments, the method further includes co-administering an agent other than a CNREB-1 inhibitor. Preferred non-CNREB-1 inhibitors include diuretics, antiadrenergic agents, vasodilators, calcium entry blockers, and angiotensin-converting enzyme inhibitors, in amounts effective to decrease CNREB-1 activity in the subject. Any of the foregoing embodiments may further comprise co-administering an adenylate cyclase inhibitor in an amount effective to decrease CNREB-1 activity in the subject.

According to another aspect of the invention, a method for increasing CNREB-1 activity in a mammalian cell, is provided. The method involves contacting the mammalian cell with a CNREB-1 activator agent in an amount effective to increase CNREB-1 activity in the mammalian cell. In some embodiments, the step of contacting the mammalian cell with a CNREB-1 activator agent may occur *in vitro* or *in vivo*. In certain embodiments, the CNREB-1 activator

agent is a nucleic acid. In preferred embodiments, the nucleic acid is a nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In further embodiments, the CNREB-1 activator agent is a polypeptide. In preferred embodiments, the CNREB-1 activator agent is a polypeptide, or a functional fragment or variant thereof, which is an expression product of the nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6.

According to another aspect of the invention, a method for increasing CNREB-1 activity in a subject, is provided. The method involves administering to a subject in need of such treatment a CNREB-1 activator agent that increases CNREB-1 activity in an amount effective to increase CNREB-1 activity in the subject. In some embodiments, the amount is sufficient to increase CNREB-1 activity above normal baseline levels. In certain embodiments the subject has a condition that includes hypotension, impotence, spinal cord injury, toxic shock syndrome, or blood transfusion. In further embodiments, the CNREB-1 activator agent is a nucleic acid. In preferred embodiments, the nucleic acid is a nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In yet further embodiments, the CNREB-1 activator agent is a polypeptide. In preferred embodiments, the CNREB-1 activator agent is a polypeptide, or a functional fragment or variant thereof, which is an expression product of the nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In some embodiments, the CNREB-1 activator agent is administered acutely. In certain embodiments, the CNREB-1 activator agent is administered prophylactically. In still further embodiments, the method further includes co-administering an agent other than a CNREB-1 activator agent. Preferred non-CNREB-1 activator agents include flurocortisone, potassium tablets, vasopressin analogues, somatostatin analogues, beta-blockers, sympathomimetics, dopamine antagonists, venoconstrictors, and adenylate cyclase activators.

According to another aspect of the invention, a method for determining the level of CNREB-1 expression in a subject, is provided. The method involves: (a) obtaining a test sample from the individual; (b) measuring the level of expression of CNREB-1 in the test sample; and (c) comparing the measured level of expression of CNREB-1 to a control, to determine the level

of CNREB-1 expression in the subject. In some embodiments, the expression of CNREB-1 in step (b) is CNREB-1 mRNA expression. In certain embodiments, the expression of CNREB-1 in (b) is CNREB-1 polypeptide expression. In still further embodiments, the test sample is tissue or a biological fluid. For methods in which CNREB-1 mRNA expression is measured, preferred assays include nucleic acid amplification assays and hybridization assays such as northern blotting. For methods in which CNREB-1 polypeptide expression is measured, preferred assays include using monoclonal antisera to CNREB-1 or polyclonal antisera to CNREB-1. In further embodiments, an increase in the level of CNREB-1 expression compared to the control is indicative of the subject's susceptibility to developing a renin-angiotensin system mediated disorder. In certain embodiments, the renin-angiotensin system mediated disorder includes an elevated risk of hypertension, hypertension, an elevated risk of myocardial infarction, an elevated risk of an ischemic stroke, ischemic stroke, an elevated risk of a condition selected from the group consisting of progressive renal disease, nephropathy, congestive heart failure, coronary artery disease, optic disc swelling, and coarctation of the aorta, or has a condition selected from the group consisting of progressive renal disease, nephropathy, congestive heart failure, coronary artery disease, optic disc swelling, and coarctation of the aorta.

According to a further aspect of the invention, a method for determining a subject's susceptibility to developing a renin-angiotensin system mediated disorder, is provided. The method involves: (a) characterizing CNREB-1 nucleic acid sequences in a test sample, wherein the test sample is obtained from the subject; (b) comparing the CNREB-1 nucleic acid sequences of the test sample to CNREB-1 nucleic acid sequences of a control sample, wherein an observed alteration or match in a CNREB-1 nucleic acid sequence in the test sample as compared to the CNREB-1 nucleic acid sequences in the control sample is indicative of the subject's susceptibility to developing a renin-angiotensin system mediated disorder. In some embodiments, the observed alteration is apparent when a CNREB-1 nucleic acid sequence in the test sample is compared to wild-type CNREB-1 nucleic acid sequences in the control sample. In other embodiments, the observed match is apparent when a CNREB-1 nucleic acid sequence in the test sample is compared to mutant CNREB-1 nucleic acid sequences in the control sample. In further embodiments, CNREB-1 mRNA molecules are compared. In yet further embodiments, alteration of CNREB-1 mRNA is detected by hybridization of mRNA from the test sample to a CNREB-1 nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ

ID NO:6. In some embodiments, CNREB-1 cDNA sequences are compared and the comparing is performed by hybridization of a CNREB-1 cDNA probe to genomic DNA isolated from the test sample. In certain embodiments, the method further comprises: (a) subjecting genomic DNA isolated from a test sample of the subject to Southern hybridization with the CNREB-1 cDNA probe; and (b) comparing the hybridizations of: (i) the CNREB-1 cDNA probe to a test sample of the subject and (ii) the CNREB-1 cDNA probe to a control sample. In any of the foregoing embodiments, the CNREB-1 cDNA probe detects a restriction fragment length polymorphism. In yet further embodiments, CNREB-1 nucleic acid sequences are compared, the comparing being performed by determining the sequence of at least a portion of a CNREB-1 cDNA in the test sample using a polymerase chain reaction, deviations in the CNREB-1 cDNA determined from that of the wild-type CNREB-1 nucleic acid sequence shown in SEQ ID NO:1, is indicative of the subject's susceptibility to developing a renin-angiotensin system mediated disorder. Renin-angiotensin system mediated disorders are as described above.

In other embodiments, the alteration of CNREB-1 nucleic acid sequences is detected by identifying a mismatch between molecules (a) a CNREB-1 cDNA or CNREB-1 mRNA isolated from the test sample, and (b) a nucleic acid probe complementary to the human wild-type CNREB-1 nucleic acid sequence, when molecules (a) and (b) are hybridized to each other to form a duplex. In some embodiments, CNREB-1 nucleic acid sequences are compared and the alteration of CNREB-1 nucleic acid sequences is detected by the steps of: (a) amplifying CNREB-1 cDNA sequences in the test sample, and (b) hybridizing the amplified CNREB-1 cDNA sequences to nucleic acid probes which comprise CNREB-1 sequences.

In yet further embodiments, CNREB-1 nucleic acid sequences are compared and the alteration of CNREB-1 nucleic acid sequences is detected by molecular cloning of CNREB-1 genes in the test sample and sequencing all or part of the cloned CNREB-1 gene.

In any of the foregoing embodiments, detection of alteration of CNREB-1 nucleic acid sequences includes screening for a deletion mutation, a point mutation, and/or an insertion mutation. The test sample may be tissue, tissue biopsy and/or a biological fluid. In preferred embodiments, the test sample may be obtained from tissues of a subject that include brain, heart, breast, colon, bladder, uterus, prostate, stomach, testis, ovary, pancreas, pituitary gland, adrenal gland, thyroid gland, salivary gland, mammary gland, kidney, liver, intestine, spleen, thymus, bone marrow, trachea, and lung. In further preferred embodiments, the test sample may be obtained from a biological fluid of a subject that includes amniotic fluid, aqueous humor, bile,

blood, bronchoalveolar lavage, bronchial fluid, cerebrospinal fluid, follicular fluid, gingival crevicular fluid, middle ear fluid, peritoneal fluid, pleural fluid, prostatic fluid, saliva, seminal fluid, serum, sweat, synovial fluid, tears, culture supernatants, and urine.

According to another aspect of the invention, a method for modulating c-myc expression in a cell, is provided. The method involves contacting a cell expressing c-myc with a CNREB-1 activity modulator agent in the cell in an amount effective to modulate c-myc expression in the cell. In some embodiments, the step of contacting the mammalian cell expressing c-myc with a CNREB-1 activity modulator agent may occur *in vitro* or *in vivo*. In certain embodiments, the CNREB-1 activity modulator agent is a nucleic acid. In further embodiments, the CNREB-1 activity modulator agent is a polypeptide.

According to another aspect of the invention, a method for modulating collagen Type II expression in a cell, is provided. The method involves contacting a cell expressing collagen Type II with a CNREB-1 activity modulator agent in the cell in an amount effective to modulate collagen Type II expression in the cell. Preferred conditions and CNREB-1 activity modulator agents are as described above in the modulation of c-myc expression.

According to a further aspect of the invention, a method for modulating T cell Receptor expression in a cell, is provided. The method involves contacting a cell expressing T cell Receptor with a CNREB-1 activity modulator agent in the cell in an amount effective to modulate T cell Receptor expression in the cell. Preferred conditions and CNREB-1 activity modulator agents are as described above in the modulation of c-myc expression.

Pharmaceutical compositions also are provided in some aspects of the invention. A pharmaceutical composition of the invention includes a CNREB-1 inhibitor in a pharmaceutically effective amount to inhibit CNREB-1 activity, and a pharmaceutically acceptable carrier. In certain embodiments, the pharmaceutical composition, further comprises an adenylate cyclase inhibitor in a pharmaceutically effective amount to inhibit CNREB-1 activity. In any of the foregoing embodiments, the CNREB-1 inhibitor is a nucleic acid. In preferred embodiments, the nucleic acid is an antisense nucleic acid or a dominant negative nucleic acid of a CNREB-1 nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6.

In another aspect of the invention, a method is provided for screening for CNREB fragments and CNREB variants which bind CNRE. In another aspect of the invention, a method is provided for screening CNREB functional fragments and CNREB functional variants which

alter expression of renin. In still another aspect of the invention, a method is provided for screening for molecules which interfere with the binding in a cell, *in vitro* or *in vivo*, of native CNREB to CNRE. Assays for screening for such molecules are described in the detailed description below. The invention also embraces products identified by the foregoing screening processes, including functional fragments and functional variants of CNREB-1 and CNREB-2 polypeptides that bind to CNRE and can modulate, by interfering with or by promoting, expression of renin, c-myc, collagen type II, and T cell Receptor.

According to still another aspect of the invention, the use of the foregoing molecules and compositions in the preparation of medicaments, particularly for the treatment of the aforementioned diseases, is provided.

The invention also embraces functional variants and equivalents of all of the molecules described above.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Drawings

Figure 1. Figure 1A is a Northern blot hybridization analyses of the levels of LXRα (CNBRE-1) mRNA transcripts in various tissues from adult C57BL/6J mouse; Figure 1B depicts the specific binding of LXRα (CNBRE-1) to mouse renin CNRE.

Figure 2. Construction of LXRα stable transfectants of As4.1 cells. Figure 2 A: is a schematic diagram of a LXRα (CNBRE-1) stable expression vector; Figure 2B is a Northern blot analysis of LXRα in As4.1 stable cells.

Figure 3. A Northern blot showing that cAMP induces renin expression through CNREB-1.

Figure 4. Gel mobility-shift assays showing specific binding of LXRα to human renin CNRE.

Figure 5. Bar Graph showing that LXRα confers the cAMP induced the human renin gene transactivation by cAMP stimulation.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence of the mouse CNREB-1 cDNA.

SEQ ID NO:2 is the predicted amino acid sequence of the translation product of mouse

CNREB-1 cDNA.

SEQ ID NO:3 is the nucleotide sequence of the largest open reading frame of the mouse CNREB-1 cDNA of SEQ ID NO:1 encoding for the polypeptide of SEQ ID NO:2.

SEQ ID NO:4 is the nucleotide sequence of EST AA415858.

SEQ ID NO:5 is the nucleotide sequence of GenBank Acc. No. U11685.

SEQ ID NO:6 is the nucleotide sequence of GenBank Acc. No. U22662.

SEQ ID NO:7 is the nucleotide sequence of the mouse CNREB-2 cDNA.

SEQ ID NO:8 is the predicted amino acid sequence of the translation product of mouse CNREB-2 cDNA.

SEQ ID NO:9 is the nucleotide sequence of the largest open reading frame of the mouse CNREB-2 cDNA of SEQ ID NO:7 encoding for the polypeptide of SEQ ID NO:8.

SEQ ID NO:10 is the nucleotide sequence of EST AA756195.

SEQ ID NO:11 is the nucleotide sequence of EST AA619904.

SEQ ID NO:12 is the nucleotide sequence of EST W63814.

SEQ ID NO:13 is the nucleotide sequence of EST AA014739.

SEQ ID NO:14 is the nucleotide sequence of EST W64822.

SEQ ID NO:15 is the nucleotide sequence of EST C78804.

SEQ ID NO:16 is the nucleotide sequence of EST C80984.

SEQ ID NO:17 is the nucleotide sequence of the mouse CNREB-2S cDNA (short isoform).

SEQ ID NO:18 is the predicted amino acid sequence of the translation product of mouse CNREB-2S cDNA (short isoform).

SEQ ID NO:19 is the nucleotide sequence of the mouse CNREB-2L cDNA (long isoform).

SEQ ID NO:20 is the predicted amino acid sequence of the translation product of mouse CNREB-2L cDNA (long isoform).

SEQ ID NO:21 is the nucleotide sequence of the mouse CNRE.

SEQ ID NO:22 is the nucleotide sequence of the rat CNRE.

SEQ ID NO:21 is the nucleotide sequence of the human CNRE.

Detailed Description of the Invention

One aspect of the invention involves the cloning of cDNAs encoding CNRE-binding

transcriptional factors. CNRE-binding transcriptional factors bind to any of the sequences set forth in SEQ ID NO:21, SEQ ID NO:22, or SEQ ID NO:23, commonly present in the in the 5'-flanking region (otherwise known as the promoter) of a number of genes including the human, mouse and rat renin genes (Burt DW, *et al.*, *J Biol Chem*, 1989, 264:7357-62), the human c-myc gene (Hay N, *et al.*, *Genes Dev*, 1989, 3:293-303), type II collagen, and T cell Receptor, and modulate the expression of these genes.

According to the invention, CNREB-2 is a novel CNREB transcription factor with an amino acid sequence set forth in SEQ ID NO:18 and as defined in the claims, and which is encoded also by sequences that hybridize under stringent conditions to SEQ ID NO:7, SEQ ID NO:17, and/or SEQ ID NO:19. CNREB-2 binds to the 5'-flanking region of the renin gene and modulates expression of the renin gene. The sequence of the mouse gene is presented as in any one of SEQ ID NO:7, SEQ ID NO:17, or SEQ ID NO:19 sequence, and the predicted amino acid sequence of this gene's protein product is presented as SEQ ID NO:8, SEQ ID NO:18, and SEQ ID NO:20 respectively. Preferably, CNREB-2 is the polypeptide encoded by the nucleic acid of SEQ ID NO:17, and has a predicted amino acid sequence of SEQ ID NO:18. This represents a short (CNREB-2S) isoform. This short isoform exhibits the stronger binding to CNRE of the renin promoter when compared to the other two isoforms. Analysis of the sequence by comparison to nucleic acid and protein databases show that CNREB-2 shares no significant homology with any other known gene or protein. Sequence analysis reveals that CNREB-2 is a novel zinc-finger transcriptional factor.

Also according to the invention, CNREB-1 is another CNRE-Binding transcription factor, encoded by SEQ ID NO:1. CNREB-1 also binds to the 5'-flanking region of the renin gene and also modulates expression of the renin gene. The sequence of the mouse gene is presented as SEQ ID NO:1, and the predicted amino acid sequence of this gene's protein product is presented as SEQ ID NO:2. Analysis of the sequence by comparison to nucleic acid and protein databases show that CNREB-1 shares high homology to the rattus norvegicus orphan receptor RLD-1 (SEQ ID NO:5, 93% homology at the nucleotide level), and to the human nuclear orphan receptor LXR-α (SEQ ID NO:6, 89% homology at the nucleotide level).

The invention in one aspect involves isolated nucleic acids and polypeptides. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical

synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art.

As used herein with respect to polypeptides, the term "isolated" means separated from its native environment in sufficiently pure form so that it can be manipulated or used for any one of the purposes of the invention. Thus, isolated means sufficiently pure to be used (i) to raise and/or isolate antibodies, (ii) as a reagent in an assay, or (iii) for sequencing, etc.

In general, the polypeptide of the invention (and the nucleic acids that encode them) have CNREB activity can be determined in a variety of different ways. A direct measure is by experiments in which a CNREB protein or complex of CNREB proteins is contacted with any of the CNRE sequences (SEQ ID NO:21, SEQ ID NO:22, or SEQ ID NO:23), preferably SEQ ID NO:21, under conditions which permit binding of the putative CNREB protein to the CNRE. The binding experiments can assay CNREB:CNRE binding directly, or indirectly, such as in competition experiments wherein the putative CNRE is used as a competitor nucleic acid for a known CNREB:CNRE binding pair. These conditions are generally known to one of ordinary skill in the art of protein-nucleic acid binding and can be performed using routine experimentation. Certain preferred binding conditions are set forth in greater detail in the Examples below. CNREB activity also can be measured more indirectly, such as by mRNA levels or by a phenotypic response to the presence of CNREB. One phenotypic measurement is to detect in a cell (or in fluid contact the cell) renin levels, as described in greater detail below.

CNREB proteins binding the CNRE motif include CNREB-1 and CNREB-2. In cells, it is believed that other CNREB proteins bind to the CNRE motif. As shown below, in cells transfected with expression vectors encoding CNREB proteins result in modulation of expression of genes having the CNRE motif in their 5'-flanking regions. In certain embodiments, CNREB proteins bind to the CNRE motif and increase expression of genes having the CNRE motif in their 5'-flanking regions. CNREB activity also includes CNREB polypeptide binding to the CNRE

motif.

Homologs and alleles of the CNREB nucleic acids of the invention can be identified by conventional techniques. Thus, an aspect of the invention is those nucleic acid sequences which code for CNREB polypeptides and which hybridize to a nucleic acid molecule consisting of the coding region of the sequences identified herein, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, and would result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of CNREB-2 nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In general homologs and alleles typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity, in some instances will share at least 50% nucleotide identity, and/or at least 65% amino acid identity, and in still other instances will share at least 60% nucleotide identity and/or at least 75% amino acid identity to any of the CNREB-2 isoforms (SEQ ID NO:17 and SEQ ID NO:18, respectively, SEQ ID NO:19 and SEQ ID NO:20, respectively, and SEQ ID NO:7 and SEQ ID NO:8, respectively). Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention. The homology can be calculated using various, publicly available software tools developed by NCBI (Bethesda, Maryland) that can be obtained through the internet (ftp:/ncbi.nlm.nih.gov/pub/). Exemplary tools include the BLAST system

available at http://wwww.ncbi.nlm.nih.gov. Pairwise and ClustalW alignments (BLOSUM30 matrix setting) as well as Kyte-Doolittle hydropathic analysis can be obtained using the MacVetor sequence analysis software (Oxford Molecular Group). Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention.

In screening for CNREB related genes, such as homologs and alleles of CNREB-2, a Southern blot may be performed using the foregoing conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film or a phosphoimager plate to detect the radioactive signal.

Given that the expression of the CNREB-1 and CNREB-2 gene is abundant in certain human tissues, and given the teachings herein of full-length CNREB cDNA clones, other mammalian sequences such as the human cDNA clones corresponding to the mouse CNREB genes can be isolated from a cDNA library prepared from one or more of the tissues in which CNREB expression is abundant, using standard colony hybridization techniques.

The invention also includes degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating CNREB-2 polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

The invention also provides isolated unique fragments of CNREB-2 isoforms (SEQ ID NO:17 and SEQ ID NO:18, respectively, SEQ ID NO:19 and SEQ ID NO:20, respectively, and SEQ ID NO:7 and SEQ ID NO:8, respectively) or complements of thereof. A unique fragment is one that is a 'signature' for the larger nucleic acid. For example, the unique fragment is long enough to assure that its precise sequence is not found in molecules within the human genome outside of the CNREB-2 nucleic acids defined above (and human alleles). Those of ordinary skill

in the art may apply no more than routine procedures to determine if a fragment is unique within the human genome. Unique fragments, however, exclude fragments completely composed of the nucleotide sequences of any sequence selected from the sequence group consisting of: (1) sequences described in SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16, (2) complements of (1), and (3) fragments of (1) and (2), or other previously published sequences as of the filing date of this application.

A fragment which is completely composed of the sequence described in the foregoing sequence groups is one which does not include any of the nucleotides unique to the sequences of the invention. Thus, a unique fragment must contain a nucleotide sequence other than the exact sequence of those in GenBank and/or the foregoing sequence groups, or fragments thereof. The difference may be an addition, deletion or substitution with respect to the GenBank sequence or it may be a sequence wholly separate from the GenBank sequence.

Unique fragments can be used as probes in Southern and Northern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. As known to those skilled in the art, large probes such as 200, 250, 300 or more nucleotides are preferred for certain uses such as Southern and Northern blots, while smaller fragments will be preferred for uses such as PCR. Unique fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments, as demonstrated in the Examples, or for generating immunoassay components. Likewise, unique fragments can be employed to produce nonfused fragments of the CNREB-2 polypeptides, useful, for example, in immunoassays or therapeutic applications. Unique fragments further can be used as antisense molecules to inhibit the expression of CNREB-2 nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

As will be recognized by those skilled in the art, the size of the unique fragment will depend upon its conservancy in the genetic code. Thus, some regions of CNREB-2 molecules (nucleic acids and polypeptides -as described throughout the specification and in the claims) and complements, will require longer segments to be unique while others will require only short segments, typically between 12 and 32 nucleotides (e.g. 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 and 32 bases long). Virtually any segment of the region of SEQ ID NO:7 beginning at nucleotide 1 and ending at nucleotide 3,847, or SEQ ID NO:9 beginning at nucleotide 1 and ending at nucleotide 1,288, or SEQ ID NO:17 beginning at nucleotide 1 and ending at nucleotide 2,336, or SEQ ID NO:19 beginning at nucleotide 1 and ending at nucleotide

2,651, or complements thereof, that is 12 or more nucleotides in length (excluding the ones that share identity with SEQ ID NOs:10, 11, 12, 13, 14, 15 and 16) will be unique.

In the claims it is mentioned that unique fragments exclude sequences identical with certain particular nucleic acids or that are identical to only fragments thereof. It is intended that the claims not embrace such molecules which are in the prior art. Thus, the nucleic acids which consist only of these sequences, or which consist only of fragments of these sequences, are considered to be within the prior art. Nucleic acids, however, which include any portion of the novel sequence of the invention are included to be embraced by the claims, including sequences comprising contiguous portions of the novel sequences and the prior art sequences. Such sequences have unexpected properties, as described herein. In one embodiment the unique fragment does not include any portion of the excluded prior art sequences. Those skilled in the art are well versed in methods for selecting unique sequences, typically on the basis of the ability of the unique fragment to selectively distinguish the sequence of interest from non-CNREB-2 nucleic acids. A comparison of the sequence of the fragment to those on known data bases typically is all that is necessary, although *in vitro* confirmatory hybridization and sequencing analysis may be performed.

As mentioned above, the invention embraces antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding a CNREB polypeptide, to decrease CNREB activity (and in particular CNREB-1 and CNREB activity). This is desirable in virtually any medical condition wherein a reduction of CNREB activity is desirable, including certain renin-angiotensin mediated disorders. Antisense CNREB molecules, for example, can be used to slow down or arrest the proliferation of cancer cells expressing c-myc *in vivo*. When using antisense preparations of the invention, slow intravenous administration is preferred.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, theCNREBy, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the

particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon any of the CNREB cDNA sequences disclosed herein, or upon allelic or homologous genomic and/or cDNA sequences , one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nat. Med.* 1(11):1116-1118, 1995). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted by antisense oligonucleotides. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which proteins are not expected to bind. Finally, although, the present invention discloses CNREB cDNA sequences, one of ordinary skill in the art may easily derive the genomic DNAs corresponding to this sequences, both for CNREB-1 and CNREB-2. Thus, the present invention also provides for antisense oligonucleotides which are complementary to the genomic DNA corresponding to SEQ ID NO:1, SEQ ID NO:17, SEQ ID NO:19, or SEQ ID NO:7. Similarly, antisense to allelic or homologous CNREB-1 and CNREB-2 cDNAs and genomic DNAs are enabled without undue experimentation.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding CNREB (1 and 2) polypeptides, together with pharmaceutically acceptable carriers. Antisense oligonucleotides may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

The invention also involves expression vectors coding for CNREB proteins and fragments and variants thereof and host cells containing those expression vectors. Virtually any cells, prokaryotic or eukaryotic, which can be transformed with heterologous DNA or RNA and which can be grown or maintained in culture, may be used in the practice of the invention. Examples include bacterial cells such as E.coli and mammalian cells such as mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, including mast cells, fibroblasts, oocytes and lymphocytes, and they may be primary cells or cell lines. Specific examples include CHO cells and COS cells. Cell-free transcription systems also may be used in lieu of cells.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g., ß-galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription

of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding CNREB-2 polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen, Carlsbad, CA), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a

multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1α, which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer*, 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the above described, CNREB cDNA sequence containing expression vectors, to transfect host cells and cell lines, be these prokaryotic (e.g., E. coli), or eukaryotic (e.g., CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include dendritic cells, U293 cells, peripheral blood leukocytes, bone marrow stem cells and embryonic stem cells. The invention also permits the construction of CNREB (particularly for the novel CNREB-2) gene "knock-outs" in cells and in animals, providing materials for studying certain aspects of CNREB activity (including CNREB-2 activity).

The invention also provides isolated polypeptides, which include the polypeptide of SEQ ID NO:18, SEQ ID NO:20, and/or SEQ ID NO:8, and unique fragments thereof. Such polypeptides are useful, for example, to stimulate cell proliferation, to affect renin expression, to detect genes flanked with CNREs, alone or as fusion proteins to generate antibodies, as components of an immunoassay, etc.

A unique fragment of an CNREB polypeptide, in general, has the features and characteristics of unique fragments as discussed above in connection with nucleic acids. As will be recognized by those skilled in the art, the size of the unique fragment will depend upon factors such as whether the fragment constitutes a portion of a conserved protein domain. Thus, some

regions of SEQ ID NO:18, 20 and/or 8, will require longer segments to be unique while others will require only short segments, typically between 5 and 12 amino acids (e.g. 5, 6, 7, 8, 9, 10, 11 and 12 amino acids long or more, including each integer up to the full length, 434, 539 and/or 763 amino acids long, respectively). Virtually any segment of SEQ ID NO:18, 20 and/or 8, excluding the ones that share identity with it (the polypeptides encoded by the nucleic acids of SEQ ID Nos:10, 11, 12, 13, 14, 15, and 16) that is 9 or more amino acids in length will be unique. As will be recognized by those skilled in the art, the size of the unique fragment will depend upon factors such as whether the fragment constitutes a portion of a conserved protein domain. Those skilled in the art are well versed in methods for selecting unique amino acid sequences, typically on the basis of the ability of the unique fragment to selectively distinguish the sequence of interest from non-family members. A comparison of the sequence of the fragment to those on known databases typically is all that is necessary.

Unique fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the polypeptide. Functional capabilities which can be retained in a unique fragment of a polypeptide include interaction with antibodies, interaction with other polypeptides or fragments thereof, and, for one important embodiment of the invention, binding to the CNRE motif. In general, the CNRE-binding characteristics of a putative CNREB polypeptide fragment can be determined by experiments in which a CNRE is contacted with the putative CNREB-binding fragment under conditions which permit binding of the CNRE to the CNREB-binding fragment. The binding experiments can assay binding directly, or indirectly, such as in competition experiments wherein the putative CNREB polypeptide fragment is used as a competitor polypeptide for a known CNRE-CNREB binding pair. These conditions are generally known to one of ordinary skill in the art of protein-nucleic acid binding and can be performed using routine experimentation. As mentioned above, such binding (for interference with binding as in a competitive binding experiment or a test for a dominant negative polypeptide) can be assayed indirectly, such as by looking for alterations in a phenotype such as expression of renin.

The invention embraces variants of the CNREB polypeptides described above. As used herein, a "variant" of a CNREB polypeptide is a polypeptide which contains one or more modification to the primary amino acid sequence of a CNREB polypeptide. Modification to a CNREB polypeptide are typically made to the nucleic acid which encodes the CNREB polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and addition of amino acids or non-amino acid moieties. Alternatively, modifications can be made

directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the CNREB amino acid sequence.

Variants can include CNREB polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a CNREB polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encodes a CNREB polypeptide in an important embodiment preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such a hairpins or loops, which can be deleterious to expression of the variant polypeptide. They also preserve, enhance or eliminate the binding of the CNREB polypeptide to the CNRE, depending upon the utility desired.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with the desired properties. Further mutations can be made to variants (or to non-variant CNREB polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., E. coli, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a CNREB gene or cDNA clone to enhance expression of the polypeptide.

The skilled artisan will realize that conservative amino acid substitutions may be made in CNREB polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e, the variants retain the functional capabilities of the CNREB-1 and/or CNREB-2 polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al.,

eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the CNREB-2 polypeptides include conservative amino acid substitutions of SEQ ID NO:18. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Thus functionally equivalent variants of CNREB polypeptides, i.e., variants of CNREB polypeptides which retain the function of the natural CNREB polypeptides, are contemplated by the invention. Conservative or nonconservative amino-acid substitutions in the amino acid sequence of CNREB polypeptides to produce functionally active variants of CNREB polypeptides typically are made by alteration of a nucleic acid encoding CNREB polypeptides. Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a CNREB polypeptide. The activity of functionally active fragments of CNREB polypeptides can be tested by cloning the gene encoding the altered CNREB polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered CNREB polypeptide, and testing for a functional capability of the CNREB polypeptides as disclosed herein.

A functional variant of a nucleic acid, as used herein means a nucleic acid which encodes a functional variant of a CNREB polypeptide.

The invention as described herein has a number of uses, some of which are described elsewhere herein. The invention permits isolation of CNREB polypeptides. A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated CNREB molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of CNREB mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce CNREB polypeptides. Those skilled in the art also can readily follow known methods for isolating CNREB polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange

chromatography and immune-affinity chromatography. The same is the case for fragments and variants thereof.

The invention also embraces isolated peptide and nonpeptide binding agents which, for example, can be antibodies or fragments of antibodies having the ability to selectively bind to CNREB polypeptides. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an $F(ab')_2$ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined

to human FR and/or Fc/pFc' regions to produce a functional antibody. Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for $F(ab')_2$, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric $F(ab')_2$ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to CNREB polypeptides, or which interfere with binding of CNREB polypeptides and their CNRE binding partners. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form, as bacterial flagella peptide display libraries or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptides and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the CNREB-2 polypeptide or a complex of CNREB-2 and a binding partner. This process can be repeated through several cycles of reselection of phage that bind to the CNREB-2 polypeptide or complex. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the

CNREB-2 polypeptide or complex can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the CNREB-2 polypeptides. Thus, the CNREB-2 polypeptides of the invention, or a fragment thereof, or complexes of CNREB-2 and a binding partner can be used to screen peptide libraries, including phage display libraries, combinatorial libraries of chemicals, peptides and naturally occurring compounds such as carbohydrates and lipids, and to utilize computer modeling programs, to identify and select peptide binding partners of the CNREB-2 polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of CNREB-2 and for other purposes that will be apparent to those of ordinary skill in the art.

A CNREB polypeptide, or a fragment thereof, also can be used to isolate their native binding partners. Isolation of binding partners may be performed according to well-known methods. For example, isolated CNREB-2 polypeptides can be attached to a substrate, and then a solution suspected of containing an CNREB-2 binding partner may be applied to the substrate. If the binding partner for CNREB-2 polypeptides is present in the solution, then it will bind to the substrate-bound CNREB-2 polypeptide. The binding partner then may be isolated. Other proteins which are binding partners for CNREB-2, may be isolated by similar methods without undue experimentation.

The invention also provides, in certain embodiments, "dominant negative" polypeptides derived from CNREB-1 and CNREB-2 polypeptides. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative catalytically-inactive kinase which interacts normally with target proteins but does not phosphorylate the target proteins can reduce phosphorylation of the target proteins in response to a cellular signal. Similarly, a dominant negative transcription factor which binds to a promoter site in the control region of a gene but does not increase gene transcription can reduce the effect of a normal transcription factor by occupying promoter binding sites without increasing

transcription.

The end result of the expression of a dominant negative polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

The isolation of the CNREB-2 cDNA and the newly identified use for the CNREB-1 cDNA also makes it possible for the artisan to diagnose a disorder characterized by an aberrant expression of CNREB. These methods involve determining expression of the CNREB gene, and/or CNREB polypeptides derived therefrom. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labeled hybridization probes as exemplified below. In the latter situation, such determination can be carried out via any standard immunological assay using, for example, antibodies which bind to the secreted CNREB protein.

As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent. In all embodiments human CNREB molecules and human subjects are preferred.

The invention also embraces determining a subject's susceptibility to developing a renin-angiotensin system mediated disorder. Such disorders are known to those of ordinary skill in the art. The renin-angiotensin system is involved in the regulation of hemodynamics and water and electrolyte balance. Factors that lower blood volume, renal perfusion pressure, or the concentration of Na^+ in plasma tend to activate the system, while factors that increase these parameters tend to suppress its function. In determining an individual's susceptibility to developing a renin-angiotensin system mediated disorder, the level of CNREB expression in the individual is obtained. The level of CNREB expression for the individual can be obtained by numerous art recognized methods. A disorder is present or there is a higher susceptibility to developing a disorder (or lower susceptibility) if CNREB activity is abnormal.

Typically, the CNREB expression level can be determined by first obtaining a test sample

from the subject. The test sample can be tissue or biological fluid. Tissues include kidney and skin, and biological fluids include blood and urine. Both invasive and non-invasive techniques can be used to obtain such samples and are well documented in the art. At the molecular level both PCR and Northern blotting can be used to determine the level of CNREB mRNA using products of this invention described earlier, and protocols well known in the art that are found in references which compile such methods. At the protein level, CNREB expression can be determined using either polyclonal or monoclonal anti-CNREB sera in combination with standard immunological assays. The preferred methods will compare the measured level of the CNREB expression of the test sample to a control. A control can include a known amount of a nucleic acid probe, a CNREB epitope (such as a CNREB expression product), or a similar test sample of a subject with a control or 'normal' level of CNREB expression. The control can be of any art recognized type, such as reagents to be tested side by side with the test fluid or a predetermined value such as a color or a number. The control population preferably includes only individuals of a similar age group as the individual tested (e.g. within 5-10 years). The predetermined value can also be a range, for example, where the population used to obtain the CNREB expression level to be used as the control, is divided equally (or unequally) into groups, such as a low-risk group and a high-risk group, or into quadrants, the lowest quadrant being individuals with the lowest risk and the highest quadrant being individuals with the highest risk. Thus, the predetermined value can depend upon the particular population selected. For example, an apparently healthy population (no detectable disease and no prior history of disease) can have a different 'normal' range of CNREB activity levels than will a population the members of which have had a prior renin-angiotensin system disorder. Accordingly, the predetermined values selected may take into account the category in which an individual falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of ordinary skill in the art.

In one aspect, the method for determining a subject's susceptibility to developing a renin-angiotensin system mediated disorder involves: (a) characterizing CNREB-1 nucleic acid sequences in a test sample, wherein the test sample is obtained from the subject; (b) comparing the CNREB-1 nucleic acid sequences of the test sample to CNREB-1 nucleic acid sequences of a control sample, wherein an observed alteration or match in a CNREB-1 nucleic acid sequence in the test sample as compared to the CNREB-1 nucleic acid sequences in the control sample is indicative of the subject's susceptibility to developing a renin-angiotensin system mediated

disorder. In some embodiments, the observed alteration is apparent when a CNREB-1 nucleic acid sequence in the test sample is compared to wild-type CNREB-1 nucleic acid sequences in the control sample. In other embodiments, the observed match is apparent when a CNREB-1 nucleic acid sequence in the test sample is compared to mutant CNREB-1 nucleic acid sequences in the control sample. In further embodiments, CNREB-1 mRNA molecules are compared. In yet further embodiments, alteration of CNREB-1 mRNA is detected by hybridization of mRNA from the test sample to a CNREB-1 nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In some embodiments, CNREB-1 cDNA sequences are compared and the comparing is performed by hybridization of a CNREB-1 cDNA probe to genomic DNA isolated from the test sample. In certain embodiments, the method further comprises: (a) subjecting genomic DNA isolated from a test sample of the subject to Southern hybridization with the CNREB-1 cDNA probe; and (b) comparing the hybridizations of: (i) the CNREB-1 cDNA probe to a test sample of the subject and (ii) the CNREB-1 cDNA probe to a control sample. In any of the foregoing embodiments, the CNREB-1 cDNA probe detects a restriction fragment length polymorphism. In yet further embodiments, CNREB-1 nucleic acid sequences are compared, the comparing being performed by determining the sequence of at least a portion of a CNREB-1 cDNA in the test sample using a polymerase chain reaction, deviations in the CNREB-1 cDNA determined from that of the wild-type CNREB-1 nucleic acid sequence shown in SEQ ID NO:1, is indicative of the subject's susceptibility to developing a renin-angiotensin system mediated disorder. Renin-angiotensin system mediated disorders are as described above.

In other embodiments, the alteration of CNREB-1 nucleic acid sequences is detected by identifying a mismatch between molecules (a) a CNREB-1 cDNA or CNREB-1 mRNA isolated from the test sample, and (b) a nucleic acid probe complementary to the human wild-type CNREB-1 nucleic acid sequence, when molecules (a) and (b) are hybridized to each other to form a duplex. In some embodiments, CNREB-1 nucleic acid sequences are compared and the alteration of CNREB-1 nucleic acid sequences is detected by the steps of: (a) amplifying CNREB-1 cDNA sequences in the test sample, and (b) hybridizing the amplified CNREB-1 cDNA sequences to nucleic acid probes which comprise CNREB-1 sequences.

In yet further embodiments, CNREB-1 nucleic acid sequences are compared and the alteration of CNREB-1 nucleic acid sequences is detected by molecular cloning of CNREB-1

genes in the test sample and sequencing all or part of the cloned CNREB-1 gene.

In any of the foregoing embodiments, detection of alteration of CNREB-1 nucleic acid sequences includes screening for a deletion mutation, a point mutation, and/or an insertion mutation. The test sample may be tissue, tissue biopsy and/or a biological fluid. In preferred embodiments, the test sample may be obtained from tissues of a subject that include brain, heart, breast, colon, bladder, uterus, prostate, stomach, testis, ovary, pancreas, pituitary gland, adrenal gland, thyroid gland, salivary gland, mammary gland, kidney, liver, intestine, spleen, thymus, bone marrow, trachea, and lung. In further preferred embodiments, the test sample may be obtained from a biological fluid of a subject that includes amniotic fluid, aqueous humor, bile, blood, bronchoalveolar lavage, bronchial fluid, cerebrospinal fluid, follicular fluid, gingival crevicular fluid, middle ear fluid, peritoneal fluid, pleural fluid, prostatic fluid, saliva, seminal fluid, serum, sweat, synovial fluid, tears, culture supernatants, and urine.

By observed "alteration", it is meant that a CNREB-1 nucleic acid sequence in the test sample is compared to a control sequence in the control sample, with the control sequence being a wild-type CNREB-1 nucleic acid according to the invention (e.g., SEQ ID NO:1 or the human homolog), and the test sample CNREB-1 sequence is different to the control sample CNREB-1 sequence. Alternatively, an observed "match" may be detected when a CNREB-1 nucleic acid sequence in the test sample is compared to a control sequence in the control sample, with the control sequence being a mutant CNREB-1 nucleic acid, and the test sample CNREB-1 sequence is found identical to the control sample CNREB-1 sequence. Appropriate differences and/or matches in the sequences can be determined with no more than routine experimentation by those of ordinary skill in the art.

The invention also provides novel kits which could be used to measure the levels of the nucleic acids of the invention, expression products of the invention or anti-CNREB antibodies. In the case of nucleic acid detection, pairs of primers for amplifying CNREB nucleic acids can be included. The preferred kits would include controls such as known amounts of nucleic acid probes, CNREB epitopes (such as CNREB expression products) or anti-CNREB antibodies, as well as instructions or other printed material. The reagents may be packaged in containers and/or coated on wells in predetermined amounts, and the kits may include standard materials such as labeled immunological reagents (such as labeled anti-IgG antibodies) and the like. One kit is a packaged polystyrene microtiter plate coated with CNREB protein and a container containing labeled anti-human IgG antibodies. A well of the plate is contacted with, for example, serum,

washed and then contacted with the anti-IgG antibody. The label is then detected.

The invention also embraces methods for treating subjects with a renin-angiotensin system mediated condition. Such conditions include cardiovascular disorders associated with atherosclerotic disease. "Cardiovascular disorders associated with atherosclerotic disease" include hypertension, myocardial infarction, stroke, angina pectoris and peripheral arteriavascular disease.

Hypertension, or high blood pressure, signifies a sustained elevation of the arterial blood pressure above a designated level considered to be associated with an increased risk for experiencing a serious cardiovascular event. Hypertension is treated by reducing arterial pressures below the levels designated as hypertensive.

Congestive heart failure involves impaired heart pumping. It can result from many different conditions. Heart failure is present when the heart fails to pump commensurate with the requirements of the metabolizing tissues, or does so only from an elevating filling pressure.

Myocardial infarction involves interruption of blood flow to heart tissue resulting in death of a portion of heart muscle. The treatment of myocardial infarction can be for patients who have experienced a myocardial infarction or can be a prophylactic treatment. If prophylactic, then the treatment is for subjects having an abnormally elevated risk of a myocardial infarction. The criteria for such individuals is well known to those of ordinary skill in the art and reference may be made to Harrison's Principles of Experimental Medicine, 13th Edition, McGraw-Hill, Inc.

Ischemic stroke (ischemic cerebral infarction) is an acute neurologic injury that results from a decrease in the blood flow involving the blood vessels of the brain. Ischemic stroke is divided into two broad categories, thrombotic and embolic.

In particular, treatment according to the invention can reduce the brain injury that follows an ischemic stroke. Brain injury reduction, can be measured by determining a reduction in infarct size in the treated versus the control groups. Likewise, functional tests measuring neurological deficits can provid further evidence of reduction in brain injury in the treated subjects versus the controls. Thus, in the various accepted models of brain injury following stroke, a positive effect can be measured in treated subjects versus control subjects.

An important embodiment of the invention is treatment of a subject with an abnormally elevated risk of an ischemic stroke. As used herein, subjects having an abnormally elevated risk of an ischemic stroke are a category determined according to conventional medical practice. Typically, the risk factors associated with cardiac disease are the same as are associated with

stroke. The primary risk factors include hypertension, hypercholesterolemia, and smoking. In addition, atrial fibrillation or recent myocardial infarction are important risk factors.

The treatment of stroke can be for patients who have experienced a stroke or can be a prophylactic treatment. If prophylactic, then the treatment is for subjects having an abnormally elevated risk of an ischemic stroke, as described above. If the subject has experienced a stroke, then the treatment can include acute treatment. Acute treatment means administration of the agents at the onset of symptoms of the condition or at the onset of a substantial change in the symptoms of an existing condition.

Other subjects with an increased risk of experiencing a cardiovascular associated disorder are hypercholesteremic subjects and hypertriglyceridimic subjects.

Hypercholesterolemic subjects and hypertriglyceridemic subjects are associated with increased incidence of premature coronary heart disease. A hypercholesterolemic subject has an LDL level of >160 mg/dL or >130 mg/dL and at least two risk factors selected from the group consisting of male gender, family history of premature coronary heart disease, cigarette smoking (more than 10 per day), hypertension, low HDL (<35 mg/dL), diabetes mellitus, hyperinsulinemia, abdominal obesity, high lipoprotein (a), and personal history of ceCNREBrovascular disease or occlusive peripheral vascular disease. A hypertriglyceridemic subject has a triglyceride (TG) level of >250 mg/dL. Thus, a hyperlipidemic subject is defined as one whose cholesterol and triglyceride levels equal or exceed the limits set as described above for both the hypercholesterolemic and hypertriglyceridemic subjects.

The invention also involves the co-administration of agents that are not CNREB activators or inhibitors but that can act cooperatively, additively or synergistically with such activators or inhibitors of the invention to modulate expression of CNRE motif-containing genes (e.g. renin, c-myc, collagen type II and T cell receptor). According to some embodiments, a CNREB activator or inhibitor is administered substantially simultaneously with a non-CNREB activator or inhibitor to modulate expression of a CNRE motif-containing gene. By "substantially simultaneously," it is meant that the CNREB activator or inhibitor is administered to the subject in need of such treatment, close enough in time with the administration of the non-CNREB activator or inhibitor, whereby the non-CNREB activator or inhibitor may exert a potentiating effect on the expression-modulating activity (on CNRE motif-containing genes) of the CNREB activator or inhibitor. Thus, by substantially simultaneously it is meant that the CNREB activator or inhibitor is administered before, at the same time, and/or after the administration of

the non-CNREB activator or inhibitor. The CNREB activator or inhibitor can be administered as a polypeptide, and/or a nucleic acid which expresses a CNRE-Binding polypeptide. In preferred embodiments of the invention, treatment of a renin-angiotensin system mediated disorder may involve co-administration of non-CNREB activators or inhibitors that include renin-angiotensin inhibitors, together with a CNREB activator or inhibitor (preferably a CNREB inhibitor and in particular a CNREB-1 inhibitor).

An overview of the pathway for synthesis of the angiotensins *in vivo* is as follows. The process is initiated when the enzyme renin acts on angiotensinogen, a pseudoglobulin in blood plasma, to produce the decapeptide angiotensin I. Angiotensin I is converted by angiotensin converting enzyme (ACE) to angiotensin II (angiotensin-[1-8] octapeptide). The latter is an active pressor substance which has been implicated as the causative agent in several forms of hypertension in various mammalian species, e.g., humans.

Renin-angiotensin system inhibitors as defined herein are compounds that act to interfere with the production of angiotensin II from angiotensinogen or to interfere with the activity of angiotensin II. Such inhibitors are well known to those of ordinary skill in the art and include compounds that act to inhibit the enzymes involved in the production of angiotensin II, including renin and ACE. They also include compounds that act on the substrates of these enzymes and compounds that interfere with the activity of angiotensin II, once produced. Examples of classes of such compounds include antibodies (e.g. to renin or to ACE), amino acids and analogs thereof (including those conjugated to larger molecules), peptides (including peptide analogs of angiotensinogen and angiotensin I), pro-renin related analogs, phospholipids and more. Among the most potent and useful renin-angiotensin system inhibitors are renin inhibitors, ACE inhibitors and angiotensin II antagonists.

Renin inhibitors are well known and include amino acids and derivatives thereof, peptides and derivatives thereof and antibodies to renin. Examples of renin inhibitors that are the subject of United States patents are as follows: urea derivatives of peptides (U.S. 5,116,835); amino acids connected by nonpeptide bonds (U.S. 5114,937); di-and tripeptide derivatives (U.S. 5,106,835); amino acids and derivatives thereof (U.S. 5,104,869 and 5,095,119); diol sulfonamides and sulfinyls (U.S. 5,098,924); modified peptides (U.S. 5,095,006); peptidyl beta-aminoacyl aminodiol carbamates (U.S. 5,089,471); pyrolimidazolones (U.S. 5,075,451); fluorine and chlorine statine or statone containing peptides (U.S. 5,066,643); peptidyl amino diols (U.S. 5,063,208 and U.S. 4,845,079); N-morpholino derivatives (U.S. 5,055,466); pepstatin

derivatives (U.S. 4,980,283); N-heterocyclic alcohols (U.S. 4,885,292); monoclonal antibodies to renin (U.S. 4,780,401); and a variety of other peptides and analogs thereof (U.S. 5,071,837, U.S. 5,064,965, U.S. 5,063,207, U.S. 5,036,054, U.S. 5,036,053, U.S. 5,034,512, and U.S. 4,894,437).

ACE inhibitors intervene in the angiotensin (renin) angiotensin I angiotensin II sequence by inhibiting angiotensin converting enzyme and reducing or eliminating the formation of the pressor substance angiotensin II. ACE inhibitors are useful as antihypertensive agents and for treating congestive heart failure.

ACE inhibitors are well known and include amino acids and derivatives thereof, peptides including diand tri-peptides and antibodies to ACE. Classes of compounds known to be useful as ACE inhibitors include acylmercapto and mercaptoalkanoyl prolines such as captopril (U.S. Patent 4,105,776) and zofenopril (U.S. Patent 4,316,906), carboxyalkyl dipeptides such as enalapril (U.S. Patent 4,374,829), lisinopril (Id.), quinapril (U.S. Patent 4,344,949), ramipril (U.S. Patent 4,587,258), and perindopril (U.S. Patent 4,508,729), carboxyalkyl dipeptide mimics such as cilazapril (U.S. Patent 4,512,924) and benazepril (U.S. Patent 4,410,520), phosphinylalkanoyl prolines such as fosinopril (U.S. Patent 4,337,201) and trandolopril.

Angiotensin II inhibitors are compounds that interfere with the activity of angiotensin II. Angiotensin II inhibitors include angiotensin II antagonists and antibodies to angiotensin II. Preferred are the AT_1 specific antagonists. Angiotensin II antagonists are well known and include peptide compounds and nonpeptide compounds. Most angiotensin II antagonists are slightly modified congeners in which agonist activity is attenuated by replacement of phenylalanine in position 8 with some other amino acid; stability can be enhanced by other replacements that slow degeneration in vivo. Examples of angiotensin II antagonists include: peptidic compounds (e.g. saralasin, [San^1, Val^5, Ala^8] angiotensin -(1-8) octapeptide and related analogs); N-substituted imidazole-2-one (U.S. 5,087,634); imidazole acetate derivatives including 2-n-butyl-4-chloro-1-(2-chlorobenzyl) immidazole-5-acetic acid (see Wong et al., J. Pharmacol. Exp. Ther. 247 (1), 1-7 (1988)); 4, 5, 6, 7-tetrahydro-1 H-imidazo [4,5-c] pyridine-6-carboxylic acid and analogs derivatives (U.S. 4,816,463); N 2-tetrazole beta-glucuronide analogs (U.S. 5,085,992); substituted pyrroles, pyrazoles and triazoles (U.S. 5,081,127); phenyl and heterocyclic derivatives such as 1, 3-imidazoles (U.S. 5,073,566); imidazo-fused 7-member ring heterocycles (U.S. 5,064,825); peptides (e.g. U.S. 4,772,684); antibodies to angiotensin II (e.g. U.S. 4,302,386); and aralkyl imidazole compounds such as biphenyl-methyl substituted

imidazoles (e.g. EP no. 253,310, January 20, 1988). Other angiotensin II inhibitors currently being tested include ES-8891 (N-morpholinoacetyl-(-1-naphtyl)-L-alanyl-(4-thiazolyl) -L-alanyl (35, 45)-4-amino-3-hydroxy-5-cyclo-hexapenta-noyl-n-hexylamide, Sankyo Company Ltd., Tokyo, Japan), SK&F 108566 (E-?a?-2-[2-butyl-1-[(carboxyphenul)methyl] 1H-imidazol-5-yl]methylane]-2-thiophenepropanoic acid, SmithKline Beecham Pharmaceuticals, PA), Losartan (DUP 753/MK 954, DuPont Merck Pharmaceutical Co.), Remikirin (RO 42-5892, F. Hoffmann LaRoche AG), Adenosine A_2 agonists (Marion Merrell Dow) and certain nonpeptide heterocycles (G.D. Searle & Company).

Disorders such as essential hypertension, coronary artery disease and diabetes involve an increase in vascular smooth muscle tone which imposes limitations on the modulation of regional blood flow in the kidney, heart, brain and other segments of the vascular bed. Clinical and experimental observations suggest that an imbalance between locally produced Angiotensin II and nitric oxide (NO) leads to an inappropriate tone of vascular smooth muscle resulting in increased blood pressure and altered regional blood flow. Indeed, administrations of nitric oxide synthase (NOS) inhibitors or Angiotensin II increase the tone and/or contractility of vascular smooth muscle and systemic blood pressure, decrease regional blood flow to organs such as the kidney and heart. Conversely, NO, Angiotensin II antagonists, renin inhibitors, and angiotensin converting enzyme (ACE) inhibitors decrease the smooth muscle tone and increase regional blood flow to these organs, and decrease systemic blood pressure.

Other agents for reducing the risk of a cardiovascular disorder and useful with the agents of the invention include those selected from the group consisting of anti-thrombotic agents, anti-platelet agents, fibrinolytic agents, lipid reducing agents, direct thrombin inhibitors, and glycoprotein II b/IIIa receptor inhibitors.

Anti-thrombotic and/or fibrinolytic agents include Plasminogen (to plasmin via interactions of prekallikrein, kininogens, Factors XII, XIIIa, plasminogen proactivator, and tissue plasminogen activator[TPA]) Streptokinase; Urokinase: Anisoylated Plasminogen-Streptokinase Activator Complex; Pro-Urokinase; (Pro-UK); rTPA (alteplase or activase; r denotes recombinant); rPro-UK; Abbokinase; Eminase; Sreptase Anagrelide Hydrochloride; Bivalirudin; Dalteparin Sodium; Danaparoid Sodium; Dazoxiben Hydrochloride; Efegatran Sulfate; Enoxaparin Sodium; Ifetroban; Ifetroban Sodium; Tinzaparin Sodium; retaplase; Trifenagrel; Warfarin; Dextrans.

Anti-platelet agents include Clopridogrel; Sulfinpyrazone; Aspirin; Dipyridamole;

Clofibrate; Pyridinol Carbamate; PGE; Glucagon; Antiserotonin drugs; Caffeine; Theophyllin Pentoxifyllin; Ticlopidine; Anagrelide.

Lipid reducing agents include gemfibrozil, cholystyramine, colestipol, nicotinic acid, probucol lovastatin, fluvastatin, simvastatin, atorvastatin, pravastatin, cirivastatin.

Direct thrombin inhibitors include hirudin, hirugen, hirulog, agatroban, PPACK, thrombin aptamers.

Glycoprotein IIb/IIIa receptor Inhibitors are both antibodies and non-antibodies, and include but are not limited to ReoPro (abcixamab), lamifiban, tirofiban.

One preferred agent is aspirin.

The agents of the invention also may be administered with diuretics. Diuretics include: Thiazides, Furosemide, Spironolactone, Triamterine, Amiloride.

The agents of the invention also may be administered with calcium channel antagonists. Calcium channel Antagonists include: Nifedipine, Amlodipine, Felodipine XL, Isradipine, Nicardipine, Diltiazem, Verapamil.

The agents of the invention also may be administered with vasodialators. Vasodilators include: Alprostadil; Azaclorzine Hydrochloride; Bamethan Sulfate; Bepridil Hydrochloride; Buterizine; Cetiedil Citrate; Chromonar Hydrochloride; Clonitrate; Diltiazem Hydrochloride; Dipyridamole; Droprenilamine; Erythrityl Tetranitrate; Felodipine; Flunarizine Hydrochloride; Fostedil; Hexobendine; Inositol Niacinate; Iproxamine Hydrochloride; Isosorbide Dinitrate; Isosorbide Mononitrate; Isoxsuprine Hydrochloride; Lidoflazine; Mefenidil; Mefenidil Fumarate; Mibefradil Dihydrochloride; Mioflazine Hydrochloride; Mixidine; Nafronyl Oxalate; Nicardipine Hydrochloride; Nicergoline; Nicorandil; Nicotinyl Alcohol; Nifedipine; Nimodipine; Nisoldipine; Oxfenicine; Oxprenolol Hydrochloride; Pentaerythritol Tetranitrate; Pentoxifylline; Pentrinitrol; Perhexiline Maleate; Pindolol; Pirsidomine; Prenylamine; Propatyl Nitrate; Suloctidil; Terodiline Hydrochloride; Tipropidil Hydrochloride; Tolazoline Hydrochloride; Xanthinol Niacinate.

In other preferred embodiments of the invention, CNREB modulators of the invention, and in particular CNREB-1 inhibitors, may be co-administered with adenylate cyclase inhibitors. Adenylate cyclase inhibitors include H-89 (a PKA inhinitor), cilostazol, dibutyryl cAMP (dbcAMP), β-adrenergic receptor agonists, milnacipran, 2',3'-dideoxyadenosine (2',3'-DDA), 2'-Deoxyadenosine-3'-monophosphate, RMI 12330A or MDL 12330A, Prostaglandin E2, SQ 22536, Guanosine 5'-O-(2-thiodiphosphate), polyphloretin, and Bromacetyl-oxytocin.

In other preferred embodiments of the invention, CNREB modulators of the invention,

and in particular CNREB-1 activators, may be co-administered with adenylate cyclase activators. Adenylate cyclase activators include forskolin, NKH477, iloprost, 2-[(4-azido-2,6-diethyl phenyl)imino]imidazolidine, GRF, and Betazol.

The invention also contemplates methods for increasing CNREB-1 activity in a subject. The method involves administering to a subject in need of such treatment a CNREB-1 activator agent that increases CNREB-1 activity in an amount effective to increase CNREB-1 activity in the subject. In some embodiments, the amount is sufficient to increase CNREB-1 activity above normal baseline levels. "Normal baseline levels" of CNREB-1 activity can be established using one of the methods described elsewhere herein, for example, an indirect measurement such as detecting renin levels in a cell. It is known in the art that in order to obtain values for such control levels of CNREB activity, a number of age, sex, (etc.) matched normal subjects can be sampled. Once established, such control levels can be used to compare CNREB activity in a subject undergoing the foregoing therapy with a CNREB modulator (i.e., a CNREB-1 activator) to determine whether CNREB activity in the subject has equaled or has exceeded normal baseline levels. In certain embodiments the subject has a condition that includes hypotension, impotence, spinal cord injury, toxic shock syndrome, or blood transfusion. In further embodiments, the CNREB-1 activator agent is a nucleic acid. In preferred embodiments, the nucleic acid is a nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. In yet further embodiments, the CNREB-1 activator agent is a polypeptide. In preferred embodiments, the CNREB-1 activator agent is a polypeptide, or a functional fragment or variant thereof, of an expression product of the nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. When human subjects are in need of such treatment, the human homologs of the foregoing mouse sequences are administered. In some embodiments, the CNREB-1 activator agent is administered acutely. In certain embodiments, the CNREB-1 activator agent is administered prophylactically. In still further embodiments, the method further includes co-administering an agent other than a CNREB-1 activator agent. Preferred non-CNREB-1 activator agents include flurocortisone, potassium tablets, vasopressin analogues, somatostatin analogues, beta-blockers, sympathomimetics, dopamine antagonists, venoconstrictors, and adenylate cyclase activators.

According to the foregoing methods, the invention also involves the treatment of erectile

dysfunction (impotence) by influencing renin levels using CNREB related agents of the invention (e.g., CNREB-1 activators). Erectile dysfunction, or impotence, is a common disorder that occurs in more than 10 million men in the USA. Although psychogenic etiology was thought to be the primary cause of erectile dysfunction, it now is believed that underlying organic diseases are responsible for most instances of erectile dysfunction. This conceptual change is supported by the particularly high incidence of impotence in men with essential hypertension, coronary artery disease and diabetes. In addition, a major mechanism responsible for impotence is an increase in the tone and/or contractility of smooth muscle within the corpus cavernosum penis and penile arteries that impede the modulation of penile blood flow by physiologic regulators. A similar mechanism, an increased tone and/or contractility of vascular smooth muscle, impedes the modulation of blood flow in the coronary, renal, and other arteries of hypertensive, diabetic, etc., patients.

Other potential organic causes of erectile dysfunction include endocrine disorders, e.g., testicular failure and hyperprolactinemia; side effects of drugs, e.g., antiandrogens, antihypertensives, anticholinergics, antidepressants, antipsychotics, central nervous system depressants and drugs of habituation or addiction; penile diseases, e.g., Peyronie's disease, previous priapism, and penile trauma; neurological diseases, e.g., anterior temporal lobe lesions, diseases of the spinal cord, loss of sensory input, diseases of nervi erigentes, and diabetic autonomic neuropathy; and vascular diseases, e.g., essential hypertension, aortic occlusion, atherosclerotic occlusion or stenosis of the pudendal artery, venous leak, and diseases of the sinusoid spaces.

As a modified vascular tissue, the corpus cavernosum penis (ccp) produces and secretes the same range of autocrine and paracrine regulators as conventional vascular tissue. The smooth muscle tone of the ccp, however, does not appear to be regulated in the same manner as in the vascular wall. Presently it is postulated that the tone or contractility of ccp is modulated by adrenergic regulation and locally produced NO and endothelin. In the ccp, most studies have been directed to observing the relaxing effects of NO, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and parasympathetic innervation, which also have similar effects on conventional and ccp vascular smooth muscle.

Recently, it was discovered that renin-angiotensin system inhibitors, similar to the vascular tissue, the corpus cavernosum penis produces and secretes Angiotensin II, that plays an important role in modulation of the penile blood flow (PCT International Patent Application

WO97/10821). Local, intracavernosal, or systemic administration of Angiotensin II antagonists or ACE inhibitors has a powerful effect on the penile blood flow. This effect can be used to improve erectile dysfunction without the inconvenience and side effects of drugs used for intracavernosal pharmacotherapy. Such agents may therefore be co-administered with a CNREB-1 activator of the invention to improve the foregoing condition.

The invention also embraces a method for treating subjects with cancers expressing c-myc. It involves first determining whether the cancer expresses c-myc. If it does, then an effective amount of an CNREB-1 inhibitor which interferes with c-myc mediated activity can be administered to the subject in order to slow down, or inhibit, the proliferation of the cancer cells expressing c-myc. CNREB-1 inhibitors include antisense CNREB-1 nucleic acids, dominant negative CNREB-1 nucleic acids, dominant negative CNREB-1 polypeptides, anti-CNREB-1 antibodies, anti-CNREB-1 antibody fragments, and polypeptides and functional fragments or variants thereof which inhibit CNREB-1 activity. In certain embodiments, the CNREB-1 inhibitor is a nucleic acid. In preferred embodiments, the nucleic acid is an antisense nucleic acid or a dominant negative nucleic acid of a CNREB-1 nucleic acid selected from the group consisting of the nucleic acid of SEQ ID NO:1, the nucleic acid of SEQ ID NO:3, the nucleic acid of SEQ ID NO:5 and the nucleic acid of SEQ ID NO:6. The mode of administration and dosage of the agent will vary with the particular stage of the condition being treated, the age and physical condition of the subject being treated, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration, and the like factors within the knowledge and expertise of the health practioner. In preferred embodiments of the invention cancers expressing c-myc include: biliary tract cancer; brain cancer, including glioblastomas and medulloblastomas; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms, including acute lymphocytic and myelogenous leukemia; multiple myeloma; AIDS associated leukemias and adult T-cell leukemia lymphoma; intraepithelial neoplasms, including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas, including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer, including squamous cell carcinoma; ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreas cancer; prostate cancer; rectal cancer; sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma; skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer; testicular cancer, including germinal

tumors (seminoma, non-seminoma[teratomas, choriocarcinomas]), stromal tumors and germ cell tumors; thyroid cancer, including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor. In other embodiments cancers expressing c-myc are cancers aberrantly expressing CNREB factors. Aberrant expression is overexpression of a CNREB (CNREB-1 and/or CNREB-2).

In certain embodiments the agent can be administered, in combination with other anti-cancer agents such as: Acivicin; Aclarubicin; Acodazole Hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone Acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperlin; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene Hydrochloride; Bisnafide Dimesylate; Bizelesin; Bleomycin Sulfate; Brequinar Sodium; Bropirimine; Busulfan; Cactinomycin; Calusterone; Caracemide; Carbetimer; Carboplatin; Carmustine; Carubicin Hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cisplatin; Cladribine; Crisnatol Mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Dexormaplatin; Dezaguanine; Dezaguanine Mesylate; Diaziquone; Docetaxel; Doxorubicin; Doxorubicin Hydrochloride; Droloxifene; Droloxifene Citrate; Dromostanolone Propionate; Duazomycin; Edatrexate; Eflornithine Hydrochloride; Elsamitrucin; Enloplatin; Enpromate; Epipropidine; Epirubicin Hydrochloride; Erbulozole; Esorubicin Hydrochloride; Estramustine; Estramustine Phosphate Sodium; Etanidazole; Etoposide; Etoposide Phosphate; Etoprine; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; Flurocitabine; Fosquidone; Fostriecin Sodium; Gemcitabine; Gemcitabine Hydrochloride; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Interferon Alfa-2a; Interferon Alfa-2b; Interferon Alfa-n1; Interferon Alfa-n3; Interferon Beta- I a; Interferon Gamma- I b; Iproplatin; Irinotecan Hydrochloride; Lanreotide Acetate; Letrozole; Leuprolide Acetate; Liarozole Hydrochloride; Lometrexol Sodium; Lomustine; Losoxantrone Hydrochloride; Masoprocol; Maytansine; Mechlorethamine Hydrochloride; Megestrol Acetate; Melengestrol Acetate; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Metoprine; Meturedepa; Mitindomide; Mitocarcin; Mitocromin; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone Hydrochloride; Mycophenolic Acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Paclitaxel; Pegaspargase; Peliomycin; Pentamustine; Peplomycin Sulfate; Perfosfamide; Pipobroman; Piposulfan; Piroxantrone Hydrochloride; Plicamycin; Plomestane; Porfimer Sodium; Porfiromycin; Prednimustine; Procarbazine

Hydrochloride; Puromycin; Puromycin Hydrochloride; Pyrazofurin; Riboprine; Rogletimide; Safingol; Safingol Hydrochloride; Semustine; Simtrazene; Sparfosate Sodium; Sparsomycin; Spirogermanium Hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Taxanes; Tecogalan Sodium; Tegafur; Teloxantrone Hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprine; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Topotecan Hydrochloride; Toremifene Citrate; Trestolone Acetate; Triciribine Phosphate; Trimetrexate; Trimetrexate Glucuronate; Triptorelin; Tubulozole Hydrochloride; Uracil Mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine Sulfate; Vincristine Sulfate; Vindesine; Vindesine Sulfate; Vinepidine Sulfate; Vinglycinate Sulfate; Vinleurosine Sulfate; Vinorelbine Tartrate; Vinrosidine Sulfate; Vinzolidine Sulfate; Vorozole; Zeniplatin; Zinostatin; Zorubicin Hydrochloride.

Other anti-neoplastic compounds include: 20-epi-1,25 dihydroxyvitamin D3; 5-ethynyluracil; abiraterone; aclarubicin; acylfulvene; adecypenol; adozelesin; aldesleukin; ALL-TK antagonists; altretamine; ambamustine; amidox; amifostine; aminolevulinic acid; amrubicin; amsacrine; anagrelide; anastrozole; andrographolide; angiogenesis inhibitors; antagonist D; antagonist G; antarelix; anti-dorsalizing morphogenetic protein-1; antiandrogen, prostatic carcinoma; antiestrogen; antineoplaston; antisense oligonucleotides; aphidicolin glycinate; apoptosis gene modulators; apoptosis regulators; apurinic acid; ara-CDP-DL-PTBA; arginine deaminase; asulacrine; atamestane; atrimustine; axinastatin 1; axinastatin 2; axinastatin 3; azasetron; azatoxin; azatyrosine; baccatin III derivatives; balanol; batimastat; BCR/ABL antagonists; benzochlorins; benzoylstaurosporine; beta lactam derivatives; beta-alethine; betaclamycin B; betulinic acid; bFGF inhibitor; bicalutamide; bisantrene; bisaziridinylspermine; bisnafide; bistratene A; bizelesin; breflate; bropirimine; budotitane; buthionine sulfoximine; calcipotriol; calphostin C; camptothecin derivatives; canarypox IL-2; capecitabine; carboxamide-amino-triazole; carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; carzelesin; casein kinase inhibitors (ICOS); castanospermine; cecropin B; cetrorelix; chlorins; chloroquinoxaline sulfonamide; cicaprost; cis-porphyrin; cladribine; clomifene analogues; clotrimazole; collismycin A; collismycin B; combretastatin A4; combretastatin analogue; conagenin; crambescidin 816; crisnatol; cryptophycin 8; cryptophycin A derivatives; curacin A; cyclopentanthraquinones; cycloplatam; cypemycin; cytarabine ocfosfate; cytolytic factor; cytostatin; dacliximab; decitabine; dehydrodidemnin B; deslorelin; dexifosfamide; dexrazoxane; dexverapamil; diaziquone; didemnin B; didox; diethylnorspermine;

dihydro-5-azacytidine; dihydrotaxol, 9-; dioxamycin; diphenyl spiromustine; docosanol; dolasetron; doxifluridine; droloxifene; dronabinol; duocarmycin SA; ebselen; ecomustine; edelfosine; edrecolomab; eflornithine; elemene; emitefur; epirubicin; epristeride; estramustine analogue; estrogen agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorunicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imidazoacridones; imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; irinotecan; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide + estrogen + progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; lovastatin; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprocol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mismatched double stranded RNA; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; monoclonal antibody, human chorionic gonadotrophin; monophosphoryl lipid A + myobacterium cell wall sk; mopidamol; multiple drug resistance gene inhibitor; multiple tumor suppressor 1-based therapy; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyldinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone + pentazocine; napavin; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; neutral endopeptidase; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; O6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate

sodium; pentostatin; pentrozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum compounds; platinum-triamine complex; porfimer sodium; porfiromycin; propyl bis-acridone; prostaglandin J2; proteasome inhibitors; protein A-based immune modulator; protein kinase C inhibitor; protein kinase C inhibitors, microalgal; protein tyrosine phosphatase inhibitors; purine nucleoside phosphorylase inhibitors; purpurins; pyrazoloacridine; pyridoxylated hemoglobin polyoxyethylene conjugate; raf antagonists; raltitrexed; ramosetron; ras farnesyl protein transferase inhibitors; ras inhibitors; ras-GAP inhibitor; retelliptine demethylated; rhenium Re 186 etidronate; rhizoxin; ribozymes; RII retinamide; rogletimide; rohitukine; romurtide; roquinimex; rubiginone B1; ruboxyl; safingol; saintopin; SarCNU; sarcophytol A; sargramostim; Sdi 1 mimetics; semustine; senescence derived inhibitor 1; sense oligonucleotides; signal transduction inhibitors; signal transduction modulators; single chain antigen binding protein; sizofiran; sobuzoxane; sodium borocaptate; sodium phenylacetate; solverol; somatomedin binding protein; sonermin; sparfosic acid; spicamycin D; spiromustine; splenopentin; spongistatin 1; squalamine; stem cell inhibitor; stem-cell division inhibitors; stipiamide; stromelysin inhibitors; sulfinosine; superactive vasoactive intestinal peptide antagonist; suradista; suramin; swainsonine; synthetic glycosaminoglycans; tallimustine; tamoxifen methiodide; tauromustine; taxanes; tazarotene; tecogalan sodium; tegafur; tellurapyrylium; telomerase inhibitors; temoporfin; temozolomide; teniposide; tetrachlorodecaoxide; tetrazomine; thaliblastine; thalidomide; thiocoraline; thrombopoietin; thrombopoietin mimetic; thymalfasin; thymopoietin receptor agonist; thymotrinan; thyroid stimulating hormone; tin ethyl etiopurpurin; tirapazamine; titanocene dichloride; topotecan; topsentin; toremifene; totipotent stem cell factor; translation inhibitors; tretinoin; triacetyluridine; triciribine; trimetrexate; triptorelin; tropisetron; turosteride; tyrosine kinase inhibitors; tyrphostins; UBC inhibitors; ubenimex; urogenital sinus-derived growth inhibitory factor; urokinase receptor antagonists; vapreotide; variolin B; vector system, erythrocyte gene therapy; velaresol; veramine; verdins; verteporfin; vinorelbine; vinxaltine; vitaxin; vorozole; zanoterone; zeniplatin; zilascorb; zinostatin stimalamer.

Anti-cancer Supplementary Potentiating Agents: Tricyclic anti-depressant drugs (e.g., imipramine, desipramine, amitryptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine and maprotiline); non-tricyclic anti-depressant drugs (e.g., sertraline, trazodone and citalopram); Ca^{++} antagonists (e.g., verapamil, nifedipine, nitrendipine and

caroverine); Calmodulin inhibitors (e.g., prenylamine, trifluoroperazine and clomipramine); Amphotericin B; Triparanol analogues (e.g., tamoxifen); antiarrhythmic drugs (e.g., quinidine); antihypertensive drugs (e.g., reserpine); Thiol depleters (e.g., buthionine and sulfoximine) and Multiple Drug Resistance reducing agents such as Cremaphor EL. The compounds of the invention also can be administered with cytokines such as granulocyte colony stimulating factor.

The invention also embraces, methods for modulating the expression of CNRE-motif-containing genes. Examples of such genes include c-myc, renin, collagen Type II, and the T cell receptor. The method involves contacting a cell expressing any of the foregoing genes with a CNREB-1 activity modulator agent in the cell, in an amount effective to modulate expression of any of the foregoing genes in the cell. In some embodiments, the step of contacting the mammalian cell expressing any of the foregoing genes with a CNREB-1 activity modulator agent may occur *in vitro* or *in vivo*. In certain embodiments, the CNREB-1 activity modulator is a nucleic acid. In further embodiments, the CNREB-1 activity modulator is a polypeptide. Although CNREB-1 has been described above as the exemplary modulator, CNREB-2 can also be used. By "modulation" it is meant that an activity modulator agent can cause an increase or decrease in the expression of a CNRE-motif-containing gene. One of ordinary skill in the art would be able to determine such modulator's affect. Typically, a CNREB-1 (or -2) activator would cause an increase in the expression of a CNRE-motif-containing gene in a specific cell type. Conversely, a CNREB-1 (or -2) inhibitor would cause a decrease in the expression of a CNRE-motif-containing gene in a specific cell type. Alternative modes of action for such activators and inhibitors, however, may occur and would normally depend upon the cell type where the CNRE-motif-containing gene is expressed. One of ordinary skill in the art would be well versed into such possibilities and could accommodate for the activity needed by administering a CNREB modulator according to the specifice cell types and needs.

The pharmaceutical preparations, as described above, are administered in effective amounts. The effective amount will depend upon the mode of administration, the particular condition being treated and the desired outcome. It will also depend upon, as discussed above, the stage of the condition, the age and physical condition of the subject, the nature of concurrent therapy, if any, and like factors well known to the medical practitioner. For therapeutic applications, it is that amount sufficient to achieve a medically desirable result. In some cases this is a decrease in the blood pressure. In other cases, it is a decrease in the expression levels of renin. In still other cases, it is a decrease in c-myc expression or cell proliferation.

Generally, doses of active compounds of the present invention would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable. A variety of administration routes are available. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule. When peptides are used therapeutically, in certain embodiments a desirable route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing peptides are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712; incorporated by reference). Those of skill in the art can readily determine the various parameters and conditions for producing antibody or peptide aerosols without resort to undue experimentation.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active agent. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Lower

doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

The CNREB polypeptides or fragments thereof may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

CNREB polypeptides preferably are produced recombinantly, although such polypeptides may be isolated from biological extracts. Recombinantly produced CNREB polypeptides include chimeric proteins comprising a fusion of a CNREB protein with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, sequence specific nucleic acid binding (such as GAL4), enhancing stability of the CNREB polypeptide under assay conditions, or providing a detectable moiety, such as green fluorescent protein. A polypeptide fused to a CNREB polypeptide or fragment may also provide means of readily detecting the

fusion protein, e.g., by immunological recognition or by fluorescent labeling.

Various techniques may be employed for introducing nucleic acids of the invention into cells, depending on whether the nucleic acids are introduced *in vitro* or *in vivo* in a host. Such techniques include transfection of nucleic acid-$CaPO_4$ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid of the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of a CNREB modulator agent of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to:(a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates

at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

Examples

Experimental Procedures

Identification of CNREB-1

Using the yeast one-hybrid cloning system to screen a mouse kidney yeast library for renin CNREBs, we obtained a ~1.5 kb double positive cDNA clone designated as KND9. The KND9 cDNA clone did not contain the complete open reading frame (ORF), missing the first ATG. Blasting the nucleotide sequence of KND9 (SEQ ID NO:1) to the mouse EST cDNA database in GenBank (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast), the 282 bp 5'-end of KND9 was found to be identical to the 3'-end of a mouse EST clone (AA415858- SEQ ID NO:4). EST AA415858 is a 488 bp cDNA from mouse 2-cell embryonic library.

Two primers (P1: nucleotides 1-22 of SEQ ID NO:4, and P2: complementary sequence of nucleotides 300-322 of KND9-SEQ ID NO:1) were used for RT-PCR. 5 mg total RNAs from DBA/2J mouse kidney was used. Standard procedures were employed in reverse transcribing the cDNA. A 528 bp single band was observed using RT-PCR. Cloning and sequencing of this band revealed that the EST-AA415858 (SEQ ID NO:4) clone was the 5'-end of KND9 (SEQ ID NO:1).

Sequence analysis of CNREB-1 cDNA revealed that it encodes a 445 amino acid protein (SEQ ID NO:2) which is 97% identical to rat RLD-1 (SEQ ID NO:5) (Apfel R, et al., *Mol Cell Biol*, 1994, 14:7025-7035). CNREB-1 cDNA also contains a 84 bp 5' untranslated region (UTR), a 1335 bp open reading frame (ORF) and a 287 bp 3-UTR that included a canonical polyadenylation signal and a tract of 41 bp poly-A tail. From these results we concluded that this cDNA encoded the full-length CNREB-1 (LXRα) protein. The ORF encodes a 445 amino acid protein (M_r 50,000) that has been confirmed using *in vitro* transcription/translation. Western blot analysis demonstrated that a CNREB-1 monoclonal antibody specifically recognized *in vitro*

translated CNREB-1.

Identification of CNREB-2

Using the yeast one-hybrid cloning system, we also cloned a strong double positive cDNA clone which was ~0.7 kb in size. It was designated as KND42. In order to obtain the full mRNA of the KND 42 clone, a mouse kidney cDNA library was screened with the a ^{32}P-labeled 700 bp KND42 cDNA. We obtained 4 positive clones from 10^6 plaques after three screening. The longest one 2.4kb in size, was designated as CNREB-2 and analyzed by sequencing. CNREB-2 appeared to be a novel gene. Using this 2.4 kb cDNA as a probe for Northern-blot hybridization, we showed that a ~3.5 kb CNREB-2 mRNA is widely expressed in all of tissues examined, but a ~2.0 kb band was found to be expressed only in the intestine, kidney and liver. We postulated that the two different CNREB-2 mRNAs resulted by the alternative splicing of its mRNA or encoded by different genes. Sequencing revealed that the 5'-end of CNREB-2 was still missing. In order to obtain the remaining part of the molecule a mouse genomic library was screened for CNREB-2 using the 2.4kb size fragment. Three positive clones were identified. Further sequencing analysis revealed two alternatively spliced isoforms. A short isoform designated CNREB-2S (SEQ ID NO:17), and a long isoform designated CNREB-2L (SEQ ID NO:19) encoding, respectively, the polypeptides with the sequences set forth in SEQ ID NO:18 and SEQ ID NO:20. No homologies were found in the non-redundant GenBank gene database.

Expression of CNREB-1 in a Prokaryotic Vector

We cloned the CNREB-1 cDNA into two different expression vectors: pcDNA3.1 (Invitrogen, Carlsbad,CA) and pGEM3Zf (Promega, Madison, WI). In vitro transcription and translation assays revealed that a major 50 kDa protein and two small ones (45 kDa & 40 kDa) were produced by both CNREB-1 expression plasmids (See Figure 1). We presumed that the 45 and 40 kDa were generated by using internal in frame ATGs for translation initiation. To verify the specific binding between CNRE and CNREB-1, we performed gel-shift assays by using ^{32}P-labeled Ren1-CNRE and CNREB-1 from *in vitro* transcription and translation. We demonstrated the complex formation between CNRE and CNREB-1. Interestingly, CNREB-1 complexes were also formed with human c-myc which shares the CNRE consensus element with Ren-1CNRE and mouse renin CRE. No complex formation was found between the MHC-1 CNRE and CNREB-1.

All of these results are consistent with our previous observations (Barrett, G, et al., *Proc Acad Natl Sci USA,* 1992, 89:885-889; Horiuchi M, et al., *J Clin Invest*, 1993, 92:1805-1811).

Transient Transfection and Reporter Gene Assays

The AS4.1 cell line was derived from a renin-expressing kidney tumor induced by SV40 T-antigen mediated tumorigenesis in transgenic mice. The line was obtained from ATCC (American Tissue Culture Collection, Manassas, VA). AS4.1 cells express high levels of renin mRNA. AS4.1 cells were therefore used as a model for investigating renin gene expression.

The HeLa cell line (epithelial, cervix carcinoma) was also purchased from ATCC. HeLa cells express high levels of c-myc mRNA. HeLa cells were therefore used as a model for investigating c-myc gene expression.

All of cDNAs (CNREB-1, and controls) were cloned into eukaryotic expression vector pcDNA3.1 (Invitrogen, CA) for transfection. Transfection were performed using Fugene-6, a transfection reagent (Boehringer Mannheim, Germany), and according to the manufacturers protocols.

When the cells reached 80% confluence in 6-well cell culture plate, 1 μg of each plasmid and 0.2 μg of a β-galactosidase plasmid together with 4 μl Fugene-6 were used for each well. The β-galactosidase plasmid was used as a control for the transfection efficiency. Total RNAs were isolated by Trizol Reagent (GIBCO BRL, Gaithersburg, MD), at 18 hours after the transfection. The reporter gene assays were done at 36 hours after the transfection according to manufacturers instructions. Both of luciferase and chloramphenicol acetyltranferase (CAT) activities were performed by the assay kits from Promega, Madison, WI. The activity of each plasmid were normalized by β-galactosidase transfection efficiency.

Northern Blot Hybridization Analysis

10 μg of total RNA from different transfected samples described above were separated in 1.0% agarose gels containing 2.6 M formaldehyde and vacuum-blotted to a Zetaprobe membrane. The membrane was prehybridized in a solution containing 10% formaimide, 3x SSPE (1X = 0.18 M NaCl, 0.01 M Na_2HPO4, 1mM EDTA), 0.1% SDS, 0.5% Blotto and 0.5 mg/ml denatured herring sperm DNA for 4 h at 55°C. Hybridization was performed in a similar solution containing 10% dextran sulfate and [a-^{32}P]-dATP-labelled (NEN Dupont, MA; specific activity 3,000 Ci/mmol) fragment from the mouse renin cDNA or the c-myc cDNA (Hay N, et al., *Genes & Dev,* 1989,

3:293-303) for 12 h at 55°C. Hybridizations were performed in a hybridization incubator (Model 400, Robbins Scientific, Sunnyvale, CA). The final stringent wash was done at 65°C in 0.1X SSC (1X = 0.15 M NaCl, 0.015 M Na citrate), 1%SDS for 30 minutes. The molecular size of detected mRNA was determined from the mobility of a co-electrophoresed 0.24-9.49 Kb RNA ladder (BRL, Gaithersburg, MD).

Results

CNREB-1(LXRα) expression

Total RNA, isolated from the heart, kidney, liver, intestine, lung, and spleen was electrophoretically separated on 1.0% formadehyde/agarose gel. The RNA was transferred to a Zetaprobe membrane and hybridized with ^{32}P-labeled CNREB-1 (hereinafter used interchangeably as LXRα) (upper panel) and GAPDH (lower panel) cDNAs. Using the 1.7 kb LXRα cDNA as a probe, we performed Northern-blot hybridization to examine the tissue distribution of LXRα. LXRα mRNA is ~1.8 kb in size and is expressed in heart, kidney, liver, intestine, lung and spleen (Figure 1A).

CNREB-1 specifically binds to the putative mouse renin CNRE.

Based on its isolation via the yeast one-hybrid system, we could tentatively identify CNREB-1 as a putative mouse renin CNRE binding protein. To examine this in more detail, gel mobility shift assays using ^{32}P-labeled mouse renin CNRE as a probe and LXRα proteins produced by *in vitro* transcription/translation system were performed and revealed a specific complex formation (Figure 1B). Gel mobility-shift assays were carried out using 2.0 µl aliquots of the LXRα_produced by the *in vitro* transcription/translation system and the ^{32}P-labeled mouse renin CNRE (2.5 x 10^4 cpm). The ^{32}P-labeled probe was indicated on the top of the figure. The name and amount of proteins added to each reaction were also listed on the top of the figure. The CNRE: LXRα complexes were dramatically reduced, in a dose-responsive manner, by addition of an LXRα monoclonal antibody (a gift from Dr. Lehmann), but remained stable following addition of 50 µg bovine serum albumin (Figure 1B).

Previous studies have shown that human LXRα could form heterodimers with RXRα, which is a universal partner of nuclear receptor heterodimers, and specifically bind to the sequence 5'-AGGTCANNNNAGGTCA-3' named DR-4; (Apfel R, et al., *Mol Cell Biol*, 1994,

14:7025-7035). Using a ^{32}P-labeled DR4 element, we confirmed specific complex formation between DR4 and LXRα/RXRα heterodimers. However, neither LXRα nor RXRα alone has specifically binding to DR4 while apparently, RXRα is not necessary for LXRα binding to the CNRE sequence.

In summary, the mouse renin CNRE is a specific cis-DNA binding element for LXRα (CNREB-1).

LXRα is a Novel cAMP Responsive Transcriptional Factor

It has been well documented that cAMP stimulates renin mRNA expression and renin secretion in kidney. Interestingly, we did not detect LXRα expression in As4.1 cells by either Northern blot or Western blot analyses. This observation is consistent with the previous report that 8-bromo-cAMP or forskolin did not affect renin mRNA levels in these cells. Therefore, we hypothesized that the absence of LXRα expression in As4.1 cells is responsible for the lack of cAMP responsiveness of the renin gene expression in As4.1 cells. To test this hypothesis, we constructed stable transfectants of As4.1 cells expressing LXRα. Briefly, the LXRα was inserted into the upstream of the encephalomyocarditis virus (EMCV) internal ribosomal entry sequence (IRES) driving GFP gene of the retroviral vector, pMX (Figure 2A). The transfected cells should encode a 4 kb transcript which contains a chimeric LXRα/GFP mRNA (Figure 2B) that encodes two separated proteins (LXRα and GFP) when it is translated. At the same time, we generated a GFP mocked stable As4.1 cells used as the control for this study. Transfectants were isolated by FACS.

Using Northern blot analyses, we revealed that the level of renin mRNA is increased by ~2 fold in LXRα-As4.1 cells under basal conditions and further induced ~2.5 fold in response to 8-bromo-cAMP stimulation (Figure 3). 10 μg RNA, isolated from the control GFP-As4.1 stable cells and LXRα-As4.1 stable cells treated with cAMP for 0, 1, 6 hrs, was electrophoretically separated on 1.0% formadehyde/agarose gel. The RNA was transferred to a Zetaprobe membrane and hybridized with mouse renin cDNA probe (upper panel). The RNA agarose gel stained with ethidium bromide is shown on lower panel. The time and the concentration of cAMP stimulation is indicated on the top of the figure. In contrast, the renin mRNA levels were not altered by cAMP in the control GFP-AS4.1 cells. This is consistent with other reports that the renin gene expression was not responsive to 8-bromo-cAMP stimulation in As4.1 cells.

Taken together, the results suggest that mouse renin CNRE exerts a positive regulatory effect on mouse renin gene expression and functions as a novel cAMP response element.

LXRα is a novel cAMP responsive transcriptional factor for human renin gene expression

We extended our studies to determine if the human CNRE can also bind LXRα. In this study, we performed gel mobility shift assays using ^{32}P-labeled human renin CNRE as a probe and LXRα proteins produced by *in vitro* transcription/translation. The results revealed a specific complex formation. The CNRE:LXRα complexes were abolished by anti-LXRα antibodies but not by a control mouse IgG (Figure 4).

We then asked if CNRE:LXRα interaction may mediate cAMP induction of the human renin gene. We cotransfected a human renin gene promoter (nt -580 to nt +16 bp) fused to the luciferase reporter gene (phRen580, a gift from Dr. Pinet) with either a control vector (pcDNA3.1) or an LXRα expression vector into As4.1 cells. As4.1 cells were cotransfected a human renin gene promoter driving luciferase (phRen580) with either pcDNA3.1 or LXRα plasmid. Afetr 48 hr of transfection, the cells were stimulated by 8-Br-cAMP at 10^{-3} M for 6 hours. The luciferase activities were expressed in relative units to pcDNA3.1 without cAMP as 100% and represented the mean of assays (±S.D., n=6) normalized by β-gal activity as an internal standard for the transfection efficiency. We demonstrated that LXRα stimulated the expression of a human renin promoter/luciferase chimeric expression vector by 1.6 fold and 3.0 fold with or without cAMP stimulation, respectively (Figure 5). This result shows that LXRα conferred human renin gene transactivation by cAMP stimulation.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. All references disclosed herein are incorporated by reference in their entirety.

What is claimed is presented below and is followed by a Sequence Listing.

We claim: